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Rapid communication

Angiotensin II-induced JNK activation is mediated by NAD(P)H oxidase in isolated rat pancreatic islets

E.S. Alves [a], A.A. Haidar [a], C.D. Quadros [a], D.S. Carvalho [a], D. Morgan [b], M.S. Rocha [b], R. Curi [b], A.R. Carpinelli [b], A.E. Hirata [a,*]

[a] Department of Physiology, Federal University of Sao Paulo-UNIFESP, São Paulo, Brazil
[b] Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil

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ABSTRACT

Angiotensin II (AII), the active component of the renin angiotensin system (RAS), plays a vital role in the regulation of physiological processes of the cardiovascular system, but also has autocrine and paracrine actions in various tissues and organs. Many studies have shown the existence of RAS in the pancreas of humans and rodents. The aim of this study was to evaluate potential signaling pathways mediated by AII in isolated pancreatic islets of rats. Phosphorylation of MAPKs (ERK1/2, JNK and p38MAPK), and the interaction between proteins JAK/STAT were evaluated. AII increased JAK2/STAT1 (42%) and JAK2/STAT3 (100%) interaction without altering the total content of JAK2. Analyzing the activation of MAPKs (ERK1/2, JNK and p38MAPK) in isolated pancreatic islets from rats we observed that AII rapidly (3 min) promoted a significant increase in the phosphorylation degree of these proteins after incubation with the hormone. Curiously JNK protein phosphorylation was inhibited by DPI, suggesting the involvement of NAD(P)H oxidase in the activation of protein.

1. Introduction

The renin–angiotensin system (RAS) plays an important role in the regulation of local and systemic blood flow and blood pressure [1]. The RAS also has paracrine and autocrine actions in various tissues and organs [2]. Angiotensin II (AII), an octapeptide hormone, acutely regulates ions and water homeostasis and causes vasoconstriction. Chronic stimulation by AII promotes cardiac and vascular hypertrophy, and influences cell growth, apoptosis, migration, inflammation and fibrosis [1]. Most of the responses to AII occurs through AT1 receptor activation (a G protein coupled receptor) that leads to phosphorylation of several tyrosine kinases including the Janus Kinase family (JAK)[3]. The downstream protein phosphorylations include: the signal transducers activators of transcription (STATs), the Mitogen Activated Protein Kinases (MAPKs), Src kinases and Focal adhesion kinase (FAK) [4,5]. In hypertension and type 2 diabetes (DM2), AII and oxidative stress activate JAK/STAT [6] and MAPK [7] molecules. AII activates the JAK/STAT mainly through AT1 receptor [8–11]. The effects mediated by AII are abolished when of the STAT1 and STAT3 proteins are inhibited [12]. The MAPKs are serine–threonine kinases that mediate intracellular signaling associated to a variety of cell functions including cell proliferation, differentiation and also cell death [13]. The family of mammalian MAPKs comprises the extracellular signal regulated kinase (ERK), p38MAPK kinase and c-Jun NH2-terminal (JNK, also known as stress-activated protein kinase or SAPK). AII activates these proteins in several tissues such as vascular smooth muscle. Activation of MAPKs has been associated to hypertension, atherosclerosis and diabetes being considered the main mechanism promoting vascular damage in these conditions.

The metabolic syndrome includes impaired in glucose metabolism, insulin resistance and beta cell dysfunction. Glucotoxicity and lipotoxicity are involved in beta cell dysfunction and seem to be associated with increased oxidative stress [14,15]. Chronic hyperglycemia causes glucotoxicity in INS-1E beta cells and increasing AT1 receptor protein expression. These changes impair glucose-stimulated insulin secretion possibly through activation of NADPH oxidase, thereby promoting oxidative stress [16]. Pancreatic beta cells are potentially susceptible to oxidative stress due to low expression of antioxidant enzymes that seem to be one of the mechanisms involved in the beta cells mass loss in diabetes [17–19]. Recently, our group demonstrated that AII promotes a large production of ROS, in a dose-dependent manner, through NAD(P)H oxidase in pancreatic islets [20]. However, the signaling pathways involved in NAD(P)H oxidase activation are not established yet. The aim of this study was then to investigate the AII-induced JAK/STAT and MAPK signaling in isolated rat pancreatic islets. The involvement of ROS was assessed by using NAD(P)H oxidase inhibitor.

* Corresponding author at: Departamento de Fisiologia, Universidade Federal de São Paulo, Rua Botucatu, 862, 5ºandar, Vila Clementino, São Paulo, Brazil, 04023–062. Tel.: +55 1155764511; fax: +55 1155759165.
E-mail address: emiko.hirata@unifesp.br (A.E. Hirata).

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2. Materials and methods

2.1. Reagents

The reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were obtained from Bio-Rad (Richmond, CA, USA). Tris, EDTA, aprotinin, PMSF, dithiothreitol (DTT), Triton X-100, Tween 20, glycerol, angiotensin II, diphenylene iodonium (DPI) and collagenase were purchased from Sigma-Aldrich (St. Louis, MO, USA). The JAK2, STAT1 and STAT3 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). JNK, pJNK, p38MAPK, phospho-p38MAPK, $ERK_{1/2}$ and phopho-$ERK_{1/2}$ antibodies were from Cell Signaling Technology Inc. (Danvers, MA, USA). The enhanced chemiluminescence reagent kit, ECL, was from GE Healthcare (Buckinghamshire, UK).

2.2. Animals

The experiments were performed following the guidelines of the Animal Research Ethics Committee of the Federal University of Sao Paulo — UNIFESP. The rats were kept in groups of five at 23 °C in a room with a light/dark cycle of 12/12 h (lights on at 07:00 h). For each experiment, 2–4 eight weeks-old female albino (150–200 g) rats, were used as indicated in the Figure legends.

2.3. Isolation of pancreatic islets

Islets of Langerhans were isolated after collagenase digestion as previously described [21]. Briefly, rat islets were isolated by distension of the pancreas via the pancreatic duct with collagenase solution (0.68 mg/mL). The pancreas was then removed and digested in a shaking water bath at 37 °C.

2.4. Analysis of DNA fragmentation in isolated pancreatic islets

Groups of 20 islets were isolated as previously described and were incubated with AII (10^{-8} M) in the presence and absence of DPI for 1 h at 37 °C. Following this process the samples were centrifuged for removal of the supernatant and islets were resuspended in hypotonic solution of propidium iodide (PI) containg 0.1% citrate sodium and 0.1% Triton X-100 following incubation for 30 min at 4 °C. Samples were analyzed in flow cytometer (FACSCalibur — Becton Dickinson, San Juan, CA, USA). Fluorescence was determined in the range of 560–680 nm. Ten thousand events per sample were acquired in histograms, and analyzed using the Cell Quest software (Becton Dickinson, San Juan, CA, USA).

2.5. Western blotting analysis

Batches of 300 islets were incubated for 30 min at 37 °C in the Krebs-Henseleit/KH buffer (139 mM Na^+, 5 mM K^+, 1 mM Ca^{2+}, 1 mM Mg^{2+}, 124 mM Cl^-, 24 mM HCO_3^-) in the presence or absence of AII as indicated in the figure legends. Thereafter, solubilization buffer containing 100 mM Tris, pH 7.6, 1% Triton X-100, 0.01 mg/mL aprotinin, 2 mM PMSF, 10 mM Na_3VO_4, 10 mM NaF, 10 mM $Na_4P_2O_7$, and 10 mM EDTA was added and the isolated pancreatic islets were sonicated to disrupt the cells. Insoluble material was removed by 30 min centrifugation at 11,000 rpm and 4 °C. The supernatant was used for immunoprecipitation with anti-JAK2 antibody overnight. The immunocomplexes were bound to protein A-sepharose (6 MB, GE Healthcare,) following treatment with sample buffer containing 100 mM DTT and boiled for 5 min. The samples were subjected to 10% SDS-PAGE in a miniature slab gel apparatus (Mini-Protean, Bio-Rad Laboratories Inc, Richmond, USA). Electrotransfer of proteins from the gel to nitrocellulose membrane was performed for 90 min at 120 V (constant) in a miniature transfer apparatus (Mini-Protean, Bio-Rad Laboratories Inc, Richmond, USA). To reduce non-specific protein binding to the nitrocellulose membrane, the filter was preincubated for 2 h at room temperature in blocking buffer (5% nonfat dry milk, 10 mM Tris, 150 mM NaCl and 0.02% Tween 20). The nitrocellulose membrane blots were incubated overnight at 4 °C with the specific antibodies described in the figure legends diluted in blocking buffer (3% nonfat dry milk, 10 mM Tris, 150 mM NaCl and 0.02% Tween 20). After that, the membranes were incubated with secondary antibody (1:10000) conjugated to horseradish peroxidase and diluted in blocking buffer (1% nonfat dry milk, 10 mM Tris, 150 mM NaCl and 0.02% Tween 20) for 60 min. To visualize the autoradiography, enhanced chemiluminescence reagents (ECL, GE Healthcare, Buckinghamshire, UK) were used and the membranes were exposed to preflashed Kodak XAR film (Eastman Kodak, Rochester, NY). Band intensities were quantified by optical densitometry (Scion Image Software, Frederick, MD, USA) of the developed autoradiographs.

For whole tissue extracts, similar sized aliquots were subjected to SDS-PAGE and immunoblotted as previously described with the indicated antibodies in the figure legends (anti-JNK, anti-phospho-JNK, anti-p38MAPK, anti-phospho-p38MAPK, anti-$ERK_{1/2}$ and anti-phospho-$ERK_{1/2}$).

2.6. Statistical analysis

Results are presented as means ± SEM. Statistical analysis were performed using the unpaired Student's *t* test and one way ANOVA when appropriated. $P<0.05$ was considered to be statistically significant.

3. Results

3.1. DNA fragmentation

In order to evaluate the viability of pancreatic islets after AII and/or DPI treatments, we performed DNA-fragmentation assays based on PI (propidium iodide) technique as described in Materials and methods section. Fig. 1 shows the incorporation of PI on the DNA in cells isolated from the pancreatic islets incubated with 2.8 mM glucose (control) or 10^{-8} M AII in the presence or absence of DPI (10 μM). There were no significant differences in DNA integrity after incubation in the presence of AII or DPI.

3.2. JAK/STAT activation and interaction with STAT1 and STAT3 induced by AII in isolated pancreatic islets

To assess whether AII could activate the JAK/STAT in isolated pancreatic islets, groups of 300 islets were incubated with AII(10^{-8} M) for 60 min. After that, the islets were homogenized in specific extraction buffer and the homogenate was subjected to immunoprecipitation with anti-JAK2 antibody. The immunocomplexes were subjected to SDS-polyacrylamide gel and electrotransfered to nitrocelulose membranes that were incubated with anti-STAT1 and/or anti-STAT3 antibodies. AII caused a significant increase in the JAK2/STAT1 association (42%) without altering the total protein content of JAK2 (Fig. 2). Similarly, the association of JAK2/STAT3 was increased by two-fold due to AII treatment when compared to the control group (glucose 2.8 mM). There was no change in the total amount of JAK2 (Fig. 3).

3.3. Effect of AII on $ERK_{1/2}$ activation in isolated pancreatic islets

AII treatment provoked a significant increase of $ERK_{1/2}$ phosphorylation after three minutes of incubation, which returned to baseline

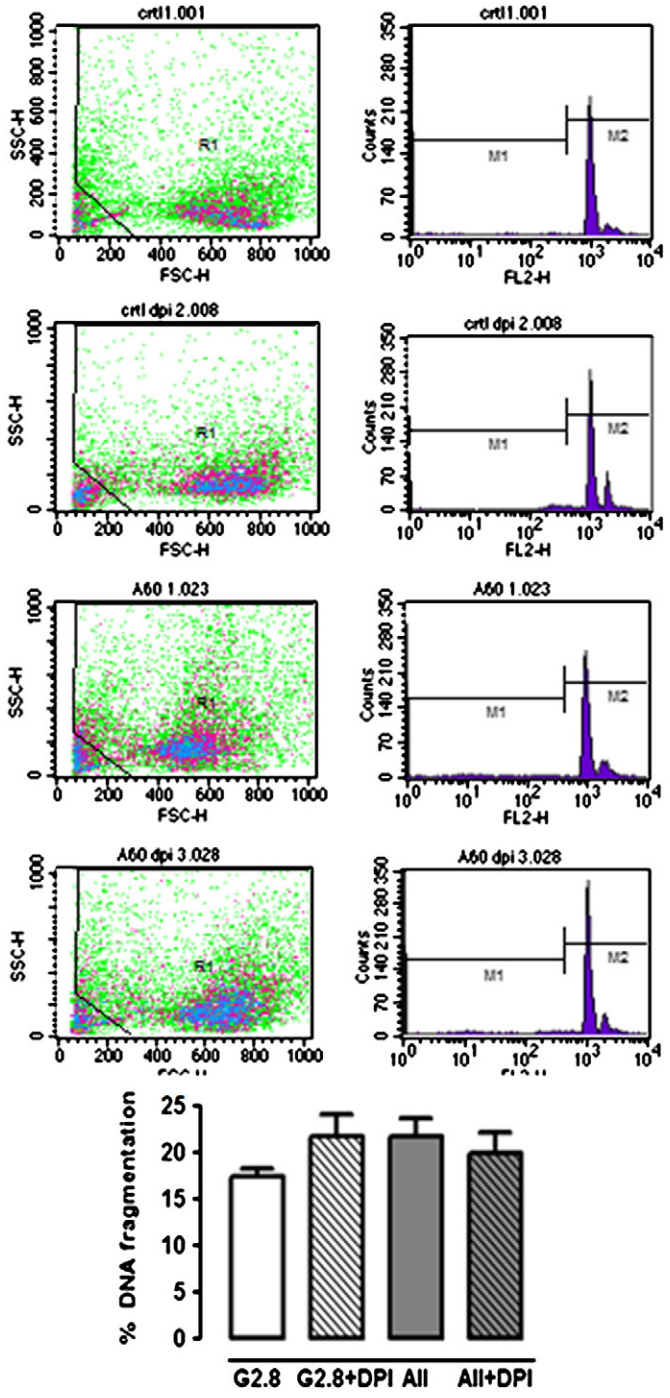

Fig. 1. Analysis of DNA fragmentation and cell death by flow cytometry. Groups of 20 pancreatic islets were isolated and incubated with 2.8 mM glucose(G) or 2.8 mM glucose plus AII 10^{-8} M (AII) in the presence or absence of DPI (10 μM). Results represent the% of cells with DNA fragmentation. Flow cytometric analysis of DNA fragmentation used propidium iodide staining. Results are expressed as mean ± SEM of 5 independent experiments. *p<0.05.

after 60 min. Treatment with DPI did not promote any change in the phosphorylation of this kinase after stimulation with AII. The total protein content also remained unchanged (Fig. 4).

3.4. Effect of AII on p38 MAPK activation in isolated pancreatic islets

After 3 min incubation in the presence of AII there was a significant increase in p38MAPK phosphorylation that returned to baseline after 60 min (Fig. 5). To determine the involvement of NAD(P)H oxidase in this effect, the samples were also incubated in the presence of 10 μM DPI, an inhibitor of this enzyme. Incubation with DPI did not alter the phosphorylation of p38MAPK induced by AII. There was no difference in the total content of p38MAPK (Fig. 5).

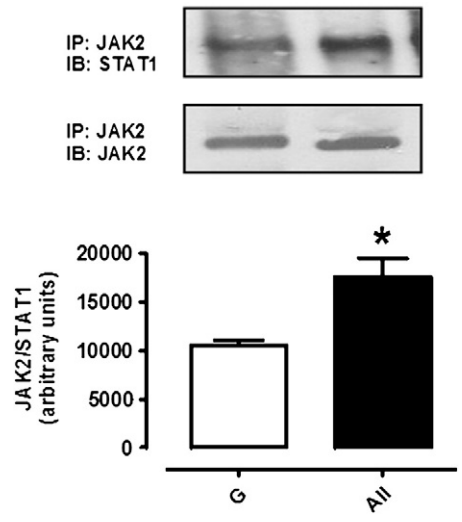

Fig. 2. AII-induced JAK2/STAT1 interaction in isolated pancreatic islets. Pancreatic islets were isolated and incubated with 2.8 mM glucose (G) or 2.8 mM glucose plus and AII 10^{-8} M (AII). After incubation, proteins were solubilized in extraction buffer and immunoprecipitated with anti-JAK2 antibody. The samples were then subjected to electrophoresis on polyacrylamide gel. After transfer to nitrocellulose membrane, immunoblotting was performed with anti-STAT1 antibody. Results are expressed as mean ± SEM of 5 independent experiments. *p<0.05.

3.5. Effect of AII on JNK activation in isolated pancreatic islets

AII treatment increased the phosphorylation of this protein at 3 min incubation that also returned to baseline after 60 min (Fig. 6). Treatment with DPI abolished the JNK phosphorylation stimulated

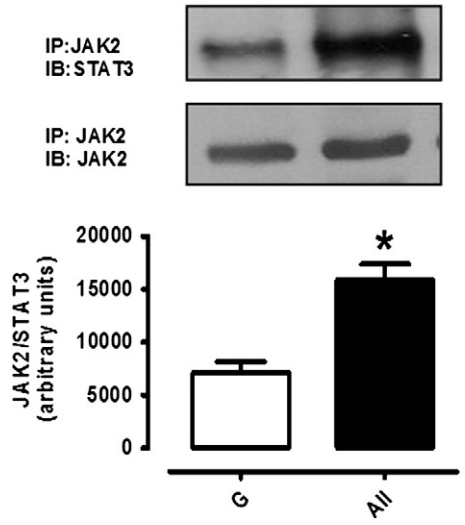

Fig. 3. AII-induced JAK2/STAT3 interaction in isolated pancreatic islets. Pancreatic islets were isolated and incubated with 2.8 mM glucose (G) or 2.8 mM glucose plus and AII 10^{-8} M (AII). After incubation, proteins were solubilized in extraction buffer and immunoprecipitated with anti-JAK2 antibody. The samples were then subjected to electrophoresis on polyacrylamide gel. After transfer to nitrocellulose membrane, immunoblotting was performed with anti-STAT3 antibody. The results are expressed as mean ± SEM of 5 independent experiments. *p<0.05.

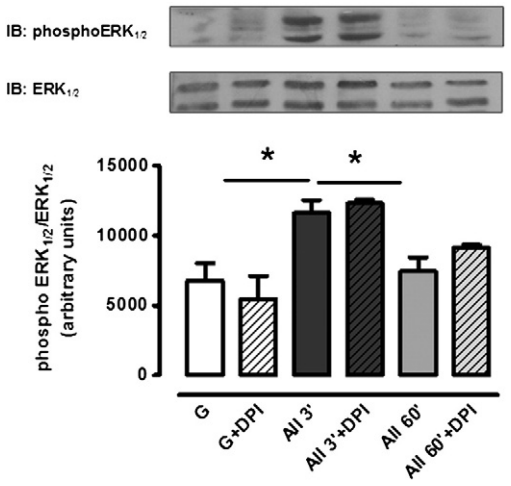

Fig. 4. AII-induced $ERK_{1/2}$ phosphorylation in isolated pancreatic islets. Pancreatic islets were isolated and incubated with 2.8 mM glucose (G) or 2.8 mM glucose plus and AII 10^{-8} M (AII) in presence or absence of DPI (10 μM). After incubation the proteins were solubilized in extraction buffer and subjected to polyacrylamide gel electrophoresis. After transfer to nitrocelulose membrane, immunoblotting was performed with anti-phopsho $ERK_{1/2}$ antibody. Phosphorylation was normalized to total $ERK_{1/2}$ protein content. The results are expressed as mean ± SEM of 5 independent experiments. *p<0.05.

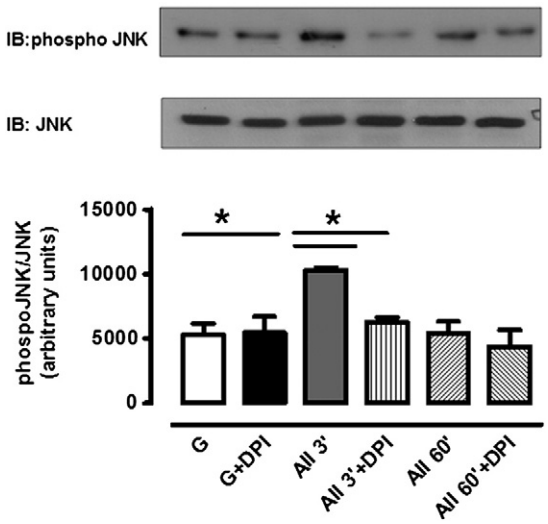

Fig. 6. AII-induced JNK phosphorylation in isolated pancreatic islets. Pancreatic islets were isolated and incubated with 2.8 mM glucose (G) or 2.8 mM glucose plus and AII 10^{-8} M (AII) in the presence or absence of DPI (10 μM). After incubation, the proteins were solubilized in extraction buffer and subjected to polyacrylamide gel electrophoresis. After transfer to nitrocellulose membrane, immunoblotting was performed with anti-phospho JNK antibody. Phosphorylation was normalized to total JNK protein content. The results are expressed as mean ± SEM of 5 independent experiments. *p<0.05.

by AII after 3 min of incubation. The total content of JNK protein remained unchanged (Fig. 6).

4. Discussion

Increased production of ROS is associated to several deleterious mechanisms and cell death. We have previously demonstrated that AII promotes increased superoxide production in pancreatic islets in a dose–response manner reaching the maximum response at 10^{-6} M. In order to avoid deleterious effects for the pancreatic islets in this study, AII concentration was set at 10^{-8} M [20]. As indicated by DNA fragmentation assays, treatment with AII and DPI did not affect pancreatic islet integrity (Fig. 1). So, the experimental protocol used allows the evaluation of the effect of supraphysiological doses of AII on signaling pathways as proposed.

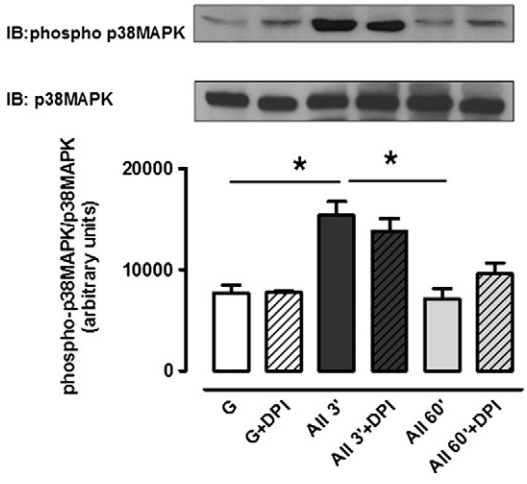

Fig. 5. AII-induced p38MAPK phosphorylation in isolated pancreatic islets. Pancreatic islets were isolated and incubated with 2.8 mM glucose (G) or 2.8 mM glucose plus and AII 10^{-8} M (AII) in presence or absence of DPI (10 μM). After incubation, the proteins were solubilized in extraction buffer and subjected to polyacrylamide gel electrophoresis. After transfer to nitrocelulose membrane, immunoblotting was performed with anti-phospho p38MAPK. Phosphorylation was normalized to total p38MAPK protein content. The results are expressed as mean ± SEM of 5 independent experiments. *p<0.05.

AII promotes JAK/STAT activation and the recruitment of members of MAPKs family including $ERK_{1/2}$, p38MAPK and JNK [22–24]. The stimulation of these pathways leads to cardiac hypertrophy, inflammation and vascular smooth muscle growth, in addition to increased oxidative stress demonstrating the potential role of AII as a pro-inflammatory agent [25–27]. In our previous study, AII led to an increase of superoxide radicals generation in isolated pancreatic islets and this effect was abolished by DPI, an inhibitor of NAD(P)H oxidase [20].

Morgan et al. [28] showed an increased expression of the components of NAD(P)H oxidase complex and subsequent increased production of superoxide radicals after treatment of pancreatic cells with an pro-inflammatory agent, IL-1b. Thus, it is possible that inflammatory stimuli such as IL-1b or AII can also activate these pathways in pancreatic islets, thereby causing oxidative stress. High content of ROS and oxidative stress are reported in pancreatic beta cells from animals with DM2 that may contribute to beta cell dysfunction [29]. Superoxide radicals can also act as second messengers and activate other proteins linked to cell growth, mitogenesis and inflammation. Similar to cytokine, AII is able to phosphorylate JAK2 protein and to increase its association with STAT proteins, which then migrates to the nucleus and activates gene expression. In the present study, AII was able to increase JAK2 association with STAT1 (Fig. 2) and also with STAT3 proteins (Fig. 3) in pancreatic islets. In isolated rat pancreatic islets, AII rapidly promotes maximum activation of NAD(P)H oxidase and subsequent increase in the production of superoxide [20] that leads to activation of MAPKs proteins [30,31]. Our results showed that AII promotes an increase in $ERK_{1/2}$, p38MAPK and JNK phosphorylation in rat pancreatic islets from.

The $ERK_{1/2}$ proteins are involved in signal transduction of several growth factors, mitogenic factors and hormones. These proteins, when activated by AII, may stimulate contraction of vascular smooth muscle and increased oxidative stress. In our study, AII caused an increase in $ERK_{1/2}$ phosphorylation after 3 min incubation, but this effect was not abolished by DPI (Fig. 4). In aorta isolated from

hypertensive rats, AII infusion lead to an increase of $ERK_{1/2}$ phosphorylation and the subsequent increase in the generation of superoxide. This effect was abolished when an inhibitor of $ERK_{1/2}$ was used, but not by apocynin or tempol treatment [32]. In the present study AII activates $ERK_{1/2}$ in isolated pancreatic islets through NAD(P)H oxidase independent mechanism (Fig. 4).

P38MAPK and JNK are activated mainly by inflammatory cytokines and cellular stressors agents being involved in activation of pathways related to apoptosis and cell death. These two proteins can also be activated by superoxide radicals derived from NAD(P)H oxidase [33,34]. AII increased the p38MAPK phosphorylation (Fig. 5) and, similarly to $ERK_{1/2}$, this effect was not prevented when the islets were pre-treated with DPI. On the other hand, AII increased JNK phosphorylation and treatment with DPI abolished this response, so NAD(P)H oxidase modulates this effect (Fig. 6).

JNK pathway is activated by various stressor signals, such as cytokines, AII and oxidative stress. The activation of JNK and oxidative stress are probably involved in the deterioration of beta cell function and in the DM2 genesis.

Forkhead box-containg protein O-subfamily (FOXO1) is a transcription factor that plays a key role in the regulation of cell cycle progression, determination of cell size, cell death and cell differentiation, stress resistance and energy metabolism [35]. In pancreas of adult animals, FOXO1 is exclusively expressed in pancreatic beta cells. It is not found in acinar cells or other cell types found in pancreas [36]. In pancreatic islets activation of FOXO1 leads to an inhibition of the transcription factor PDX-1 (pancreatic duodenal hemobox). PDX-1 plays an important role in the function and growth of beta cells. After being phosphorylated, FOXO1 migrates to the nucleus and inactivates PDX-1. Oxidative stress promotes FOXO1 translocation from the cytoplasm to the nucleus. PDX-1 in turn translocates from the nucleus to the cytoplasm leading to an inhibition of cell growth and proliferation [37,38]. After incubation with AII, no significant difference in FOXO1 phosphorylation was observed at 3 or 60 min incubations (data not shown). However we noticed a tendency of increase in FOXO1 phosphorylation at 3 min, suggesting that this pathway can also be activated by AII in isolated pancreatic islets. Our results are not in agreement with data found for FOXO1 phosphorylation through by JNK activation.

We demonstrated herein that AII activate MAPKs via activation of JAK/STAT in isolated rat pancreatic islets. Among the MAPKs assessed the activation of JNK seems to involve the participation of NAD(P)H oxidase (Fig. 7).

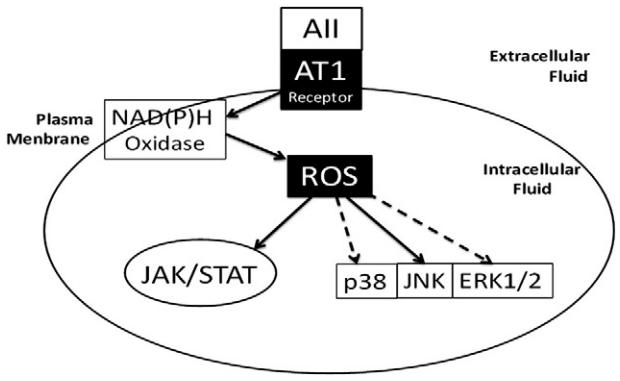

Fig. 7. Proposed model of activation of protein JAK/STAT and MAPK by Angiotensin II in isolated rat islets. AII binds to the AT1 receptor, and promotes the activation of NAD(P)H oxidase, which leads to an increase in reactive oxygen species (ROS) production ROS promote subsequent phosphorylation of JAK/STAT and JNK proteins. Regarding p38MAPK and $ERK_{1/2}$ proteins, we found that their activation does not involve NAD(P)H oxidase.

Acknowledgments

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